Food Chemistry 112 (2009) 1038–1045

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Measurement of malondialdehyde in fish: A comparison study between HPLC methods and the traditional spectrophotometric test

Rogério Mendes *, Carlos Cardoso, Carla Pestana

Department of Upgrading of Fish Products, National Institute of Biological Resources, INRB/IPIMAR, Av. Brasília, 1449-006 Lisbon, Portugal

ARTICLE INFO

Article history:
Received 5 March 2008
Received in revised form 28 May 2008
Accepted 22 June 2008

Keywords:
Malondialdehyde
Fish
Lipid oxidation
2-Thiobarbituric acid
2,4-Dinitrophenylhydrazine

ABSTRACT

Alternative methods to the traditional spectrophotometric determination of the malondialdehyde-thiobarbituric acid (MDA-TBA) complex (method A) and to the overestimation of MDA levels in the TBA reaction have been develop for the evaluation of lipid oxidation in fish. In this study, two HPLC separation methods of the MDA-TBA (method B) and MDA-dinitrophenylhydrazine (MDA-DNPH) adduct (method C) were investigated and compared to the traditional spectrophotometric TBA test (method A) in samples of chilled fish (hake, sea bream and sardine). Detection limits were 0.16, 0.10 and 0.20 μM MDA and quantification limits were 0.23, 0.17 and 0.26 μM MDA, for methods A, B and C, respectively. Recovery of method B ranged between 100% and 108% and of method C between 90% and 112%. Method A presented low recovery levels (under 71%). Overall method performance followed the order HPLC method MDA-DNPH > HPLC method MDA-TBA > traditional spectrophotometric TBA test. Though showing a better accuracy and specificity, method C had, however, some disadvantages, a relatively high limit of detection (0.20 μM MDA) and a lower reproducibility at lower MDA contents in standards and samples. Nevertheless, these are not critical drawbacks for an application in routine fish analysis, given the high MDA concentrations in oxidised fish. The application of the modified HPLC methods in fish samples with different levels of MDA, showed that these methods are useful for the samples with low amounts of oxidation products, such as chilled hake as well as in samples with high levels of oxidation, like 15 days chilled stored sardine.

1. Introduction

Lipid oxidation in food is associated with the development of rancidity and oxidative deterioration. Fish, on account of its high content of polyunsaturated fatty acids (PUFA), is highly susceptible to lipid oxidation during manipulation, processing, and cooking. As a consequence of oxidative spoilage, lipid hydroperoxides are formed, which, in turn, are unstable and decompose to aldehydes, ketones, alcohols, acids or hydrocarbons (St. Angelo, 1996). These so-called secondary oxidation products can change food quality, namely, colour, texture, flavour and odour (Fernández, Pérez-Álvarez, & Fernández-López, 1997). One of the most important products of oxidation is malondialdehyde (MDA), which is thought to be a carcinogenic initiator and mutagen. MDA has often been used as marker of oxidative damage in biological samples (Kinter, 1995) and foods (St. Angelo, 1996). The most widely used method for determination of MDA is the spectrophotometric determination of the pink fluorescent MDA-thiobarbituric acid (MDA-TBA) complex produced after reaction with 2-thiobarbituric acid (TBA) at low pH and high temperature (Bergamo, Fedele, Balestrieri, Abrescia, & Ferrara, 1998). This simple technique is used in fish analysis, whenever an assessment of lipid oxidation is required (Panpipat & Yongsawatdigul, 2008). Reaction occurs by attack of the monoenolic form of MDA on the active methylene groups of TBA. Visible and ultraviolet spectrophotometry of the pigment confirms its primary maximum at 532–535 nm and a secondary one at 245–305 nm (Sinnhuber, Yu, & Yu, 1958). The intensity of colour is a measure of MDA concentration (Tarladgis, Pearson, & Dugan, 1964) and has been correlated with rancidity (Zipser, Kwon, & Watts, 1964). Reaction kinetics depends on the concentration of TBA solution, temperature and pH (Fernández et al., 1997).

Several variations of MDA-TBA method exist, namely, a method for fish lipids was described by Ke and Woyewoda (1979) and for fish by Vyncke (1970). Furthermore, various procedures are generally performed in food: direct heating of the sample with TBA (Sinnhuber & Yu, 1958), sample distillation (Ke, Cervantes, & Robles-Martinez, 1984), lipid extraction with organic solvents (Pikul, Leszczynski, & Kummerow, 1989) or acid extraction of MDA (Squires, 1990), followed by acid reaction with TBA. In spite of these improving modifications, the traditional spectrophotometric TBA test has been criticised for its lack of sensitivity (Squires, 1990) and its high inaccuracy, since TBA reacts not only with MDA but also with many other compounds (for instance, carbohydrates, amino acids, pyridines, pigments, etc.) (Guillen-Sans & Guzman-Chozas, 1998), interfering in the TBA assay and resulting in

* Corresponding author. Tel.: +351 213027036; fax: +351 213015948.
E-mail address: rogerio@ipimar.pt (R. Mendes).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.06.052

considerable overestimation, as well, variability in the results (Mateos, Lecumberri, Ramos, Goya, & Bravo, 2005). The traditional spectrophotometric TBA test has also shown problems when used in frozen Gadidae fish species, like saithe, haddock, cod and others. During such storage, these species can produce an important amount of formaldehyde which in turn was shown to interfere in the traditional spectrophotometric TBA test (Aubourg, 1999; Careche & Tejada, 1988). Furthermore, the high temperatures (95–100 °C), extended incubation times (up to 150 min) (Sakai, Habiro, & Kawahara, 1999; Volpi & Tarugi, 1998) and strong acidic conditions (pH 1.5–3.5) commonly required for the reaction of MDA with TBA may cause an artifactual peroxidation of sample constituents even in the presence of added antioxidants. For this reason and in order to eliminate interferences in the formation of the MDA-TBA red pigment, more sensitive and advanced methods of analysis of biological matrices by capillary gas chromatography with electron-capture and mass spectrometry and liquid chromatography–mass spectrometry were developed (Cighetti, Debiasi, Paroni, & Allevi, 1999; Jardine, Antolovich, Prenzler, & Robards, 2002; Stalikas & Konidari, 2001). Also more specific high-performance liquid chromatographic (HPLC) approaches using reversed-phase chromatography (Draper & Hadley, 1990; Squires, 1990) with UV/VIS absorption (Cesa, 2004) or with fluorimetric detection (De las Heras, Schoch, Gibis, & Fischer, 2003) have been used. Derivatisation of MDA with 2,4-dinitrophenylhidrazine (DNPH) and conversion into pyrazole and hydrazone derivatives, has been also found to allow a specific estimation of MDA, particularly, if combined with HPLC separation (Mateos et al., 2005). Absorption at 310 nm is used to calculate MDA concentration. This method has been used to determine MDA levels in biological samples, such as rat and human plasma (Pilz, Meineke, & Gleiter, 2000) or rat urine (Ekström, Garberg, Egestad, & Högerg, 1988). However, it has not been applied yet to fish samples.

Though the mentioned disadvantages, conventional spectrophotometric MDA-TBA methods are preferred because of their simplicity. Therefore, development of a simple, sensitive and specific MDA-TBA method has remained a challenge. The objective of this study was to develop an improved sensitive and specific HPLC method for MDA determination in fish with different fat contents and different degrees of rancidity, using TBA or DNPH as derivatising reagents and compare these two methods with the traditional spectrophotometric technique (TBA test).

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals and reagents used were analytical grade of the highest purity. Potassium dihydrogenphosphate (KH_2PO_4), potassium hydroxide (KOH), hydrochloric acid fuming 37% (HCl), glacial acetic acid (CH_3COOH), trichloroacetic acid (TCA, CCl_3COOH), perchloric acid (PCA, $HCLO_4$), ethylenediaminetetracetic acid (EDTA, $C_{10}H_{16}N_2O_8$) and propyl gallate ($C_{10}H_{12}O_5$) were purchased from Merck (Darmstadt, Germany). 2-thiobarbituric acid (2-TBA, $C_4H_4N_2O_2S$) and 1,1,3,3-tetraethoxypropane (TEP, $C_{11}H_{24}O_4$) were from Sigma (St. Louis, MO, USA). 2,4-dinitrophenylhydrazine (DNPH, $C_6H_6N_4O_4$) was from Fluka (Deisenhofen, Germany). HPLC grade organic solvents were used: methanol (CH_3OH) and acetonitrile (CH_3CN) were from Merck (Darmstadt, Germany). Aqueous solutions were prepared with Milli-Q purified water.

2.2. Materials

Sardine (*Sardina pilchardus*) and farmed sea bream (*Sparus aurata*) were bought fresh from a local supermarket. South African hake (*Merluccius capensis*) was purchased frozen from a local fish processor and thawed overnight in a refrigerated chamber. For the study of rancidity development all fish species were kept in a refrigerated chamber (2 ± 1 °C) and analysed periodically over a period of 19 days.

2.3. Sample preparation

For MDA extraction, a portion (15 g) of minced fish was weighed in a 50 ml centrifuge tube and diluted with 30 ml of 7.5% TCA solution (7.5% (p/v) TCA, 0.1% (p/v) EDTA, 0.1% (p/v) propyl gallate). Mixture was then homogenised with a Polytron PT3000 blender for 1 min at 5000 rpm and filtered through filter paper (Whatman #1). Filtrate was centrifuged for 10 min at 6000 rpm. Supernatant (extract) was used in both TBA and DNPH methods.

2.4. Traditional spectrophotometric TBA determination of MDA-TBA (TBA Test)

This MDA determination (method A) was performed according to Vyncke's methodology (Vyncke, 1970). TEP (1,1,3,3-tetraethoxypropane) was used as the MDA standard, without hydrolysis prior to the TBA reaction. A standard curve was made from TEP diluted in 7.5% TCA solution, at concentrations 2.0, 4.0, 6.0, 8.0 and 10.0 μM. 5 ml sample supernatant, standard or blank was transferred into a screw-capped tube, 5 ml of 20 mM TBA solution was added, mixture was vigorously agitated in a vortex and placed in a boiling water bath for 60 min. After cooling, MDA-TBA complex was measured at 530 nm using an UNICAM Helyos spectrophotometer. Results were expressed as micromoles MDA present in 1 kg of muscle.

2.5. HPLC determination of MDA-TBA

HPLC separation of MDA-TBA adduct (method B) was performed according to the method described by Seljeskog, Hervig, and Mansoor (2006), with modifications in the sample deproteinisation procedure (see sample preparation, above). TEP was used as the MDA standard, without hydrolysis prior to the TBA reaction. A standard curve was made from TEP diluted in 7.5% TCA solution, at concentrations of 0.6, 1.3, 2.5, 5.0, 10.0 μM. 0.5 ml sample supernatant, standard or blank was transferred into a screw-capped tube, 1.5 ml of 40 mM TBA solution was added, mixture was vigorously agitated in a vortex and placed in a hot water bath at 97 °C for 60 min. After cooling in a freezer at −20 °C for 20 min, 3 ml methanol was added and mixed in a vortex. The resulting solution was filtered through a 0.2 μm PTFE membrane (Acrodisc® CR 25 mm Syringe Filter, PALL Life Sciences) into autosampler vials. HPLC analysis was performed using an Agilent 1100 Series system, equipped with pump, degasser, autosampler, spectrofluorimetric detector and system controller with a PC control program. Separation of MDA-TBA was done using a Phenomenex Gemini C18 column (5 μm, 150 × 4.6 mm), operated isocratically with a HPLC mobile phase pumped at 1.0 ml/min and consisting of 50 mM KH_2PO_4 buffer solution, methanol and acetonitrile in the proportion 72:17:11 (v/v). In this method, injection volume was 10 μl, sample run took 8 min and retention time for MDA-TBA was near 5.5 min. Spectrofluorimetric detector wavelengths were set at 525 nm (excitation) and 560 nm (emission). Results were expressed as micromoles MDA present in 1 kg of muscle.

2.6. HPLC determination of MDA-DNPH

MDA-DNPH adduct detection (method C) was based on the method described by Mateos et al. (2005), modified in order to ob-

tain a more practical and rapid method. TEP was used as the MDA standard, without hydrolysis prior to the TBA reaction. A standard curve was made from TEP diluted in 7.5% TCA solution, at concentrations of 0.6, 1.3, 2.5, 5.0, 10.0 μM. Sample supernatant, standard or blank was filtered through a 0.2 μm PTFE membrane (Acrodisc® CR 25 mm Syringe Filter, PALL Life Sciences) and 200 μl was transferred into an autosampler vial. Derivatisation of MDA as well MDA-DNPH complex separation and analysis were performed by an Agilent 1100 Series HPLC system, equipped with pump, degasser, autosampler, UV/VIS absorbance detector and system controller with a PC control program. Each vial was placed in the autosampler tray and using automated derivatization 20 μl of 5 mM DNPH (in 2 M HCl) solution were added. Injector drew and ejected two times 100 μl of the reactive mixture in order to achieve a good homogenisation. After waiting 10 min, 50 μl of the mixture was injected. For each injection cycle, special care was taken in washing the needle with acetonitrile. Separation of MDA-DNPH was done using a Phenomenex Gemini C18 column (5 μm, 250 × 4.6 mm), operated isocratically at 30 °C with a HPLC mobile phase pumped at 1.0 ml/min and consisting of water, acetonitrile and glacial acetic acid in the proportion 55:45:0.2 (v/v). In this method, sample run took 18 min time and retention time for MDA-DNPH was near 12.2 min. UV/VIS detector wavelength was set at 310 nm and results were expressed as micromoles MDA present in 1 kg of muscle.

2.7. Detection and quantification limits and linearity

Limits of detection (LD) and quantification (LQ) were calculated, respectively, as the mean blank or trace solution response plus 3 and 5 standard deviations of blank or trace solution response. For method A and B, 10 blank replicates were measured. In method C, 10 trace solution replicates with 0.08 μM MDA were measured, since blank had no signal. Trace solution concentration was determined by consecutive diluting the lowest standard until no signal was attained. The lowest concentration enabling a signal was considered the trace level. Concerning linearity, correlation coefficients of the regression lines from all three methods were determined.

2.8. Recovery/Accuracy

Recovery of MDA was evaluated by addition of known amounts of TEP (lower range: 0.15-0.30 μM; higher range: 2.50–5.00 μM) to the sample extracts. The amount of MDA was determined by using specific standard curves. Determinations were done in quadruplicate and each recovery value was the mean of all determinations. For all methods, usual MDA extraction procedures were followed. However, for MDA-TBA detection after HPLC separation, two extracts/sample preparation methods were compared:

(i) a direct 7.5% TCA extract treated according to method B;
(ii) a water extract (0.5 ml) treatment with 0.1125 N PCA (1.5 ml) and 40 mM TBA (1.5 ml), followed by reaction at 97 °C for 60 min and deproteinisation with 20% TCA (1.0 ml) and methanol (3.0 ml) according to the method of Seljeskog et al. (2006).

2.9. Analytical reproducibility

Standards: Within-run precision was determined for 13 aliquots of standard solutions with 5 concentrations ($n = 65$), injected on the same day. Between-run precision was calculated for 13 aliquots of standard solutions with 5 concentrations ($n = 65$), each aliquot being injected in one different day, within an 18 day interval. Coefficients of variation were obtained from the ratio of the standard deviation to the standards mean.

Samples: Within-run precision, for a same extract of hake or sea bream, was calculated for 13 aliquots of the respective extract. Within-run precision, for different extracts of hake, sea bream and sardine, was calculated for 13 aliquots of 13 different extracts in each given species. Each group of 13 aliquots was analysed on the same day. Between-run precision was determined for sea bream using eight different extracts on eight different days over a period of 11 days. Coefficients of variation were obtained from the ratio of the standard deviation to the samples mean.

2.10. Specificity

For the evaluation of the specificity of the studied methods, MDA contents in the muscle of hake, sea bream and sardine stored in a refrigerated chamber (2 ± 1 °C) were followed for 19 days by methods A, B and C. The same extracts were used for all methods. These three fish species were chosen in order to investigate the methods potential in assessing lipid oxidation of fish with different fat contents.

2.11. Statistical analysis

For each standard/sample and aliquot, all MDA determinations were done in duplicate. For comparison of lipid oxidation in different fish species over storage time, a general linear model —factorial analysis of variance (ANOVA)— was used to determine significant differences ($p \leqslant 0.05$) among methods. Multiple comparisons were done by the Tukey HSD test. All statistical treatment was done with the software STATISTICA© from StatSoft, Inc. (Tulsa, OK, USA), version 6.1, 2003.

3. Results and discussion

In order to achieve a rapid and simple MDA determination method with application in fish products, an HPLC method for the analysis of MDA-TBA in serum and plasma (Seljeskog et al., 2006) and an HPLC method for the analysis of MDA-DNPH in serum and liver (Mateos et al., 2005) were modified, namely, concerning deproteinisation. The main purpose of these modifications was to adapt techniques to routine fish analysis and to optimize experimental conditions. In this context, two alternative deproteinisation strategies were tested for TBA method (see below, Recovery/accuracy section). A fundamental objective was the development of simple methods, in accordance to the principle that sample preparation procedures should be kept as simple as possible, in order to avoid error additions and to maximize analyte recoveries (extra preparation steps should be added only if needed).

Typical chromatograms obtained from the MDA-TBA and MDA-DNPH adducts are shown in Fig. 1. The MDA-TBA and MDA-DNPH peaks were identified by the elution profile of authentic standard. The average retention time for the MDA-TBA adduct was around 5.6 min at a flow rate of 1.0 ml/min and it produced the main peak in chromatogram. On the other hand, the MDA-DNPH complex, detected near 12.3 min at a flow rate of 1.0 ml/min, was a small peak behind other peaks produced by TCA solution components and DNPH reagent. Hence, these peaks were also present in the blank (see peak near 7.5 min in Fig. 1). No interfering peak was detected in fish samples and chromatographic separation was very good for both MDA-TBA and MDA-DNPH analysis.

3.1. Detection and quantification limits and linearity

Detection limits were 0.16, 0.10 and 0.20 μM MDA and quantification limits were 0.23, 0.17 and 0.26 μM MDA, for the traditional

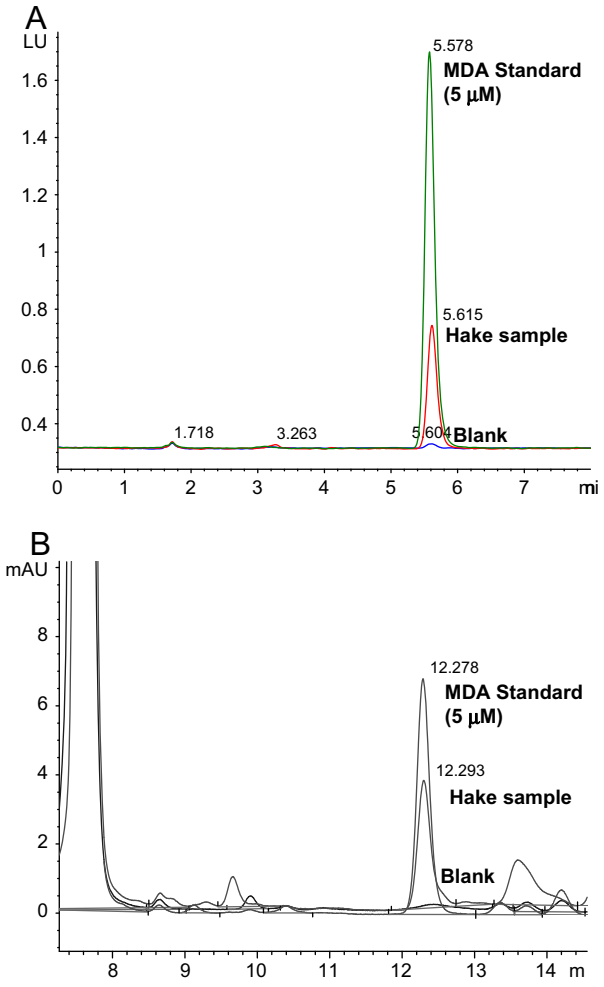

Fig. 1. A: HPLC chromatograms of MDA-TBA complex (retention time 5.6 min) in blank, MDA standard (5 μM) and hake sample (method B). B: HPLC chromatograms of MDA-DNPH complex (retention time 12.2 min) in blank, MDA standard (5 μM) and hake sample (method C).

spectrophotometric TBA test (method A) and the HPLC methods of MDA-TBA (method B) and MDA-DNPH (method C) adducts analysis, respectively. These values were well below the lowest standard concentration, 0.63 μM MDA and in view of the high MDA values typically found in fish considered suitable for the evaluation of lipid oxidation. For HPLC separation of MDA-TBA limits of detection have been reported to range between 0.015 μM (Khoschsorur et al., 2000; Tsaknis, Lalas, & Evmorfopoulos, 1999) and 1 μM or more (Kakuda, Stanley, & Van de Voort, 1981). Regarding chromatographic analysis of MDA-DNPH, limits of detection and quantification were similar to those found in other studies (Mateos et al., 2005).

In what concerns linearity, method A exhibited a linear response between 2.00 μM and 10.00 μM and a collection of 15 calibration curves presented a modest correlation coefficient, $R^2 \approx 0.90$ (data not shown). On the other hand, both HPLC methods showed a linear response of MDA in a range of concentrations from 0.63 μM to 10.00 μM and calibration curves presented high correlation coefficients $R^2 > 0.99$ ($p = 0.0001$; $n = 15$).

3.2. Recovery/Accuracy

Recovery of the samples analysed according to method B and extracted with 7.5% TCA (sample preparation method i) ranged between 100% and 108% (Table 1). This represents a significant improvement over the 66–71% recovery of MDA, when determined by the traditional spectrophotometric TBA test. Lower TBA concentration in method A (20 mM *vs* 40 mM) is a possible factor hindering recoveries near to 100%. Samples extracted with water and deproteinised after TBA derivatization (method ii) showed also after analysis with method B a reduced recovery (65–75%), presumably, because, as Pilz et al. (2000) and Bird, Hung, Hadley, and Draper (1983) refer, strong acidic conditions are essential for the release of protein bound MDA. This acid extraction was carried out in sample preparation method (i), but not in (ii), where TCA deproteinisation occurs after MDA reaction with TBA. Method C presented acceptable recovery levels in the range 90–112%. Other studies have also reported good recovery levels for DNPH HPLC method in plasma or liver samples (Mateos et al., 2005).

3.3. Analytical reproducibility

Analytical reproducibility was studied with analysis of standard solutions and sample extracts. Within-run and between-run (Table 2) precision of method B ranged, for the different standard concentrations tested, between 0.2–3.1% and 0.3–4.6%, with mean global values of 1.6% and 2.0%, respectively for within-run and between-run precision. Concerning method C, within-run and between-run (Table 2) precision ranged between 4.7–10.2% and 1.0–10.9%, with mean global values of 8.0% and 4.8%, respectively. The traditional spectrophotometric TBA test presented coefficients of variation (data not shown) ranging from 1.0% to 4.3%, being the highest percentage attained with a between-run analysis of a 2.0 μM solution.

Comparison of methods B and C shows that a higher precision was reached with method B, which can be related to the small chromatographic area of the MDA-DNPH peak (Fig. 1) and, thus, to a greater proximity of the concentration of the tested solutions to the limit of quantification (see above) of method C. However, high coefficients of variation were also determined for higher concentrations of MDA-DNPH in the within-run experiment which can be credited to the instability of the signal observed after prolonged equipment operation (≈after 15 h). For between-run precision, method C performance was more similar to method B and, moreover, coefficients of variation declined from 10.9% for lowest standard concentration to 1.0% for highest, thus, reinforcing the limit of quantification vicinity hypothesis. Standard precision values for MDA-TBA determination were comparable to those mentioned in literature (Khoschsorur et al., 2000). On the other hand lower coefficients of variation, not exceeding 5%, have been reported by other authors working on the DNPH method and on the range 0.2–20.0 μM (Mateos et al., 2005).

Within-run and between-run precision of method B results determined with hake and sea bream extracts are presented in Table 3. Coefficients of variation ranged from 2.1% for 13 within-run aliquots of the same hake extract to 9.1% for 8 different sea bream extracts in different runs. Regarding method C, coefficients of variation ranged from 5.4% for 13 within-run aliquots of the same sea bream extract to 31.6% for 13 different sea bream extracts within the same run (Table 3). In comparison to the above precision values, the traditional spectrophotometric TBA test showed a within-run coefficient of variation of the same hake extract of 14.0% (MDA content averaged 7.33 μmol/kg; data not shown). As with standard solutions, within-run trial reproducibility of method C determined with different fish extracts was much worse than in between-run situation, thus, suggesting a run duration effect similar to the one observed in the standards precision study. However, this effect was magnified by the vicinity of the method quantification limit, since the sea bream samples analysed had very low MDA contents (below 2 μmol/kg). The importance of this factor is

Table 1
Recovery data of MDA in fish samples extracts by different analytical methods*

Analytical method	MDA in sample (μM MDA ± SD)	MDA added (μM MDA)	MDA detected (μM MDA ± SD)	Recovery(%)
Method A	0.075 ± 0.011	0.15	0.175 ± 0.04	66
	0.075 ± 0.011	0.30	0.289 ± 0.13	71
Method B (i)	0.098 ± 0.019	0.15	0.249 ± 0.023	100
	0.098 ± 0.019	0.30	0.430 ± 0.004	108
Method B (ii)	0.440 ± 0.004	0.15	0.538 ± 0.047	65
	0.440 ± 0.004	0.30	0.667 ± 0.030	75
Method C	4.395 ± 0.478	2.50	6.651 ± 0.146	90
	4.395 ± 0.478	5.00	10.013 ± 0.480	112

* Presented values correspond to mean ± standard deviation (n = 4).
(i) Sample extraction with 7.5% TCA.
(ii) Sample extraction with water and deproteinisation with 20% TCA + PCA 0.1125 N (Seljeskog et al., 2006).

Table 2
Reproducibility data (precision within run and from run to run) for the HPLC determinations of the MDA-TBA and MDA-DNPH complex in standard solutions

	Expected 0.625 μM	Expected 1.250 μM	Expected 2.500 μM	Expected 5.000 μM	Expected 10.000 μM
	Measured (μM)	Measured (μM)	Measured (μM)	Measured (μM)	Measured (μM)
Within run					
Method B: MDA-TBA					
Mean	0.585	1.172	2.468	5.083	10.034
SD ±	0.018	0.030	0.012	0.010	0.160
CV(%)	3.1	2.5	0.5	0.2	1.6
Method C: MDA-DNPH					
Mean	0.637	1.276	2.516	4.906	10.039
SD ±	0.055	0.131	0.159	0.229	1.006
CV(%)	8.6	10.2	6.3	4.7	10.0
From run to run					
Method B: MDA-TBA					
Mean	0.645	1.201	2.447	4.992	10.014
SD ±	0.030	0.025	0.040	0.070	0.032
CV(%)	4.6	2.1	1.6	1.4	0.3
Method C: MDA-DNPH					
Mean	0.576	1.253	2.548	5.015	9.983
SD ±	0.063	0.045	0.097	0.245	0.100
CV(%)	10.9	3.6	3.8	4.9	1.0

highlighted when a comparison is made between sea bream and highly oxidised sardine samples (MDA above 300 μmol/kg, requiring a 1:10 dilution). Within-run coefficient of variation for different extracts was in this case quite lower for sardine (9.6% *vs* 31.6%). This MDA concentration effect was almost absent for method B (3.0 *vs* 3.5%), although contents range was not so wide. Method B reproducibility was in a precision range comparable to that found by other authors using also HPLC separation of MDA-TBA in different biological material, albeit with some technical differences (Khoschsorur et al., 2000; Knight, Smith, Kinder, & Pieper, 1988). Variation coefficients of up to 5.6% (between-run), for very low MDA levels (0.74 μmol/kg) were also reported by authors working on HPLC separation of MDA-TBA in fish samples (Bergamo et al., 1998). Method C precision was not so good as reported in the literature for an equivalent DNPH derivatization method (Fenaille, Mottier, Turesky, Ali, & Guy, 2001), where, for milk powder (4–9 μmol MDA/kg), variation coefficients of up to 3% were calculated. Comparison of methods B and C with the traditional spectrophotometric technique shows that for MDA concentrations in the same range both HPLC methods B and C have greater reproducibility (2.1% and 5.4% *vs* 14.0%: data not shown). Analysis of the data shows that both methods have adequate reproducibility for application in routine fish analysis.

3.4. Methods comparison and specificity

Methods comparison and evaluation of potential in assessing different levels of lipid oxidation was carried out during chilled storage of three fish species with different fat content, hake (0.6 g fat/100 g fresh weight), sea bream (9.7 g/100 g) and sardine (14.3 g/100 g). In what concerns specificity, methods A, B and C presented parallel MDA variations during storage time (Fig. 2). The only exception was MDA content determined by method A in sardine from day 15. However, MDA concentrations were substantially different between techniques. In hake and sea bream, method C produced significantly lower ($p < 0.05$) MDA contents than methods A and B. In fact, after day 8 in hake and day 4 in sea bream, a gap was formed between method C values and the others and the difference progressively widened. This suggests that other compounds released by further fish spoilage reacted with TBA, while DNPH remained specific and only reacted with MDA. In sea bream, after day 6, whereas MDA measured by methods A and B suffered only minor fluctuations, method C variations were greater, reaching 0.9 μmol MDA/kg on 11th day.

For sardine and most of the time, method C mean values were lower. The considerable production of MDA in sardine possibly reduces the effect of other thiobarbituric acid reactive substances, thus, narrowing the difference between TBA and DNPH techniques. With respect to former techniques (methods A and B), there were significant differences ($p < 0.05$) only in sea bream and higher values were determined by MDA-TBA HPLC method. This may be unexpected since HPLC should separate MDA-TBA from other components that interfere in the traditional spectrophotometric TBA test by also absorbing at 530 nm. Though, almost no such component was found in method B chromatograms (see Fig. 1). A plausible explanation for the difference may be related to the low recovery values found for method A (see above recovery/accuracy). This can possibly also explain the relatively low MDA content mea-

Table 3
Reproducibility data (precision within run and from run to run) for the HPLC determinations of the MDA-TBA and MDA-DNPH complex in hake, sea bream and sardine samples

	Hake Same extract (μmol MDA/ kg)	Hake Different extract (μmol MDA/ kg)	Sea bream Different extract (μmol MDA/ kg)	Sardine Different extract (μmol MDA/ kg)
Within run				
Method B: MDA-TBA				
Mean	19.89	19.95	7.29	
SD ±	0.41	0.54	0.25	
CV(%)	2.1	3.0	3.5	
Method C: MDA-DNPH				
Mean	7.08		1.81	335.55
SD ±	0.38		0.57	32.38
CV(%)	5.4		31.6	9.6
From run to run				
Method B: MDA-TBA				
Mean			7.47	
SD ±			0.68	
CV(%)			9.1	
Method C: MDA-DNPH				
Mean			2.30	
SD ±			0.26	
CV(%)			11.4	

sured by method A in sardine on 15th day. The consistently lower values estimated by method C and the fact that these are not linked to any recovery problems, show that both methods based on the quantification of the MDA-TBA adduct (methods A and B) may overestimate MDA and are therefore less accurate. Overestimation may result from TBA's low specificity, but, also, from an artefactual peroxidation of sample constituents (Mateos et al., 2005) even in the presence of added antioxidants (EDTA and propyl gallate), as a consequence of TBA reaction requiring treatment at high temperatures for extended incubation times.

Similar conclusions have been reported by other authors comparing TBA and DNPH techniques in other kinds of sample, be it milk powder (Fenaille et al., 2001), MDA estimates reduced up to 17-fold with DNPH, or plasma and tissues (Türközkan, Erdamar, & Seven, 2006), reductions up to 100-fold. However, MDA content values were not so drastically reduced by DNPH technique in fish samples. Probably, the cause for this lies much more in the greater levels of lipid oxidation in fish, conducive to stronger MDA production, than in lower artefactual MDA formation or any MDA overestimation by method C in fish samples.

In comparison with published data, MDA levels in hake and sardine were relatively high (particularly, exceeding 29 μmol MDA/kg hake on 8th day and 700 μmol MDA/kg sardine on 15th day). Nevertheless, about 100 μmol MDA/kg was reported for hake stored in ice (Ruiz-Capillas & Moral, 2001). Concerning sardine, in spite of being a much fatter fish and, thereby, more prone to oxidation, literature values determined by classical spectrophotometric technique (TBA test) in iced whole sardine reached 250 μmol MDA/kg flesh after 14 days (Nunes, Batista, & Campos, 1992) or, only, 111 μmol MDA/kg after nine days (Aubourg, Sotelo, & Gallardo, 1997). However, Chaijan, Benjakul, Visessanguan, and Faustman (2006) reported over 8000 μmol MDA/kg in sardine muscle after 15 days. This wide range may arise from different biological condition of fish, *post-mortem* handling before storage in ice and, also, from a different method used by the latter authors, since ground sample was homogenised with a solution containing TBA and TCA. This simultaneous derivatisation and deproteinisation procedure promotes TBA reaction with other sample components besides MDA and, thus, may lead to overestimated MDA contents.

For all methods, MDA content in fish samples varied during chilled storage in a broadly typical way for this oxidation product, namely, an initial phase of MDA content increase followed by a later phase of decreasing MDA levels in, which the interaction between MDA and nucleophilic compounds included in fish muscle (namely, protein-type compounds) led to the formation of tertiary oxidation products. Similar changes in MDA content were reported by Ozden, Inugur, and Erkan (2007) and Mendes and Gonçalves (in press) in sea bass and sea bream, by Rodriguez-Casado et al. (2007) in sardine and by Ruiz-Capillas, Saavedra, and Moral (2003) in hake. However, in hake and sea bream, all methods showed a later MDA increase (near day 15). Similar behaviour was also reported by Ruiz-Capillas and Moral (2001) in chilled hake, where a slight increasing trend of MDA content over three storage weeks was observed. Moreover, in sardine, after an initial peak on 4th day, MDA maximum was reached on 15th day. Changes during chilled storage suggest that MDA content — as peroxide value — can be the result of a balance between rates of formation and destruction reactions and, as such, the following hypothesis can be formulated: initial MDA content increases occur as lipid oxidation proceeds at slow rate, intermediate declines (as seen between 8th and 11th day in hake muscle or between 4th and 6th day in sardine) may be caused by the same modest rates of lipid oxidation coupled with increased MDA degradation, greater increases after several storage days may be due to a markedly high rate of MDA formation and, finally, a deceleration of this process brings about a final decline in MDA concentration (clearly seen in sardine after day 15). Higher rates of MDA formation may be due to greater release of free iron and other prooxidants from the muscle, which becomes increasingly degraded when storage time increases (Chaijan et al., 2006).

In order to evaluate the reliability of the HPLC methods the correlation between them was followed (Fig. 3). Whereas, correlation coefficients between method A and the HPLC methods were low (0.785 for B and 0.746 for C), correlation coefficient between methods B and C was considerably high (0.974) and similar to the ones reported between the MDA-TBA complex determined by HPLC and by the classical spectrophotometric TBA test in oxidised (0.9794) and non-oxidised (0.9819) traditional fish products (Tsaknis et al., 1999). Therefore, independent determinations by methods B and C agree to each other, being MDA variation estimated by C largely a translation to a lower range of B values (correlation slope < 1). This, in turn, reinforces the artefactual MDA formation hypothesis. On the other hand, method A deviations from methods B and C seem to highlight a greater uncertainty in classical spectrophotometric TBA technique without HPLC separation. However, standard deviations of the method A results were not particularly higher in comparison to the others (Fig. 2). It should be noticed

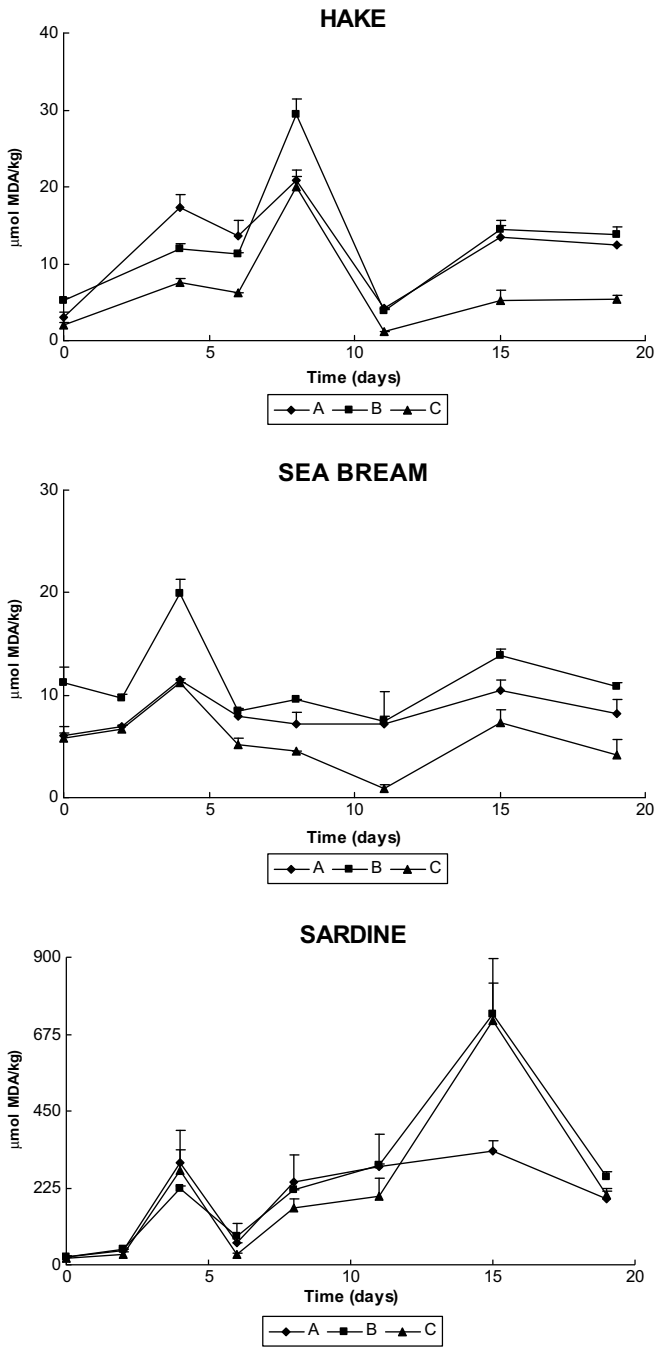

Fig. 2. Changes in MDA levels (μmol MDA/kg) of hake, sea bream and sardine during chilled storage at 2 ± 1 °C, measured by methods A, B and C.

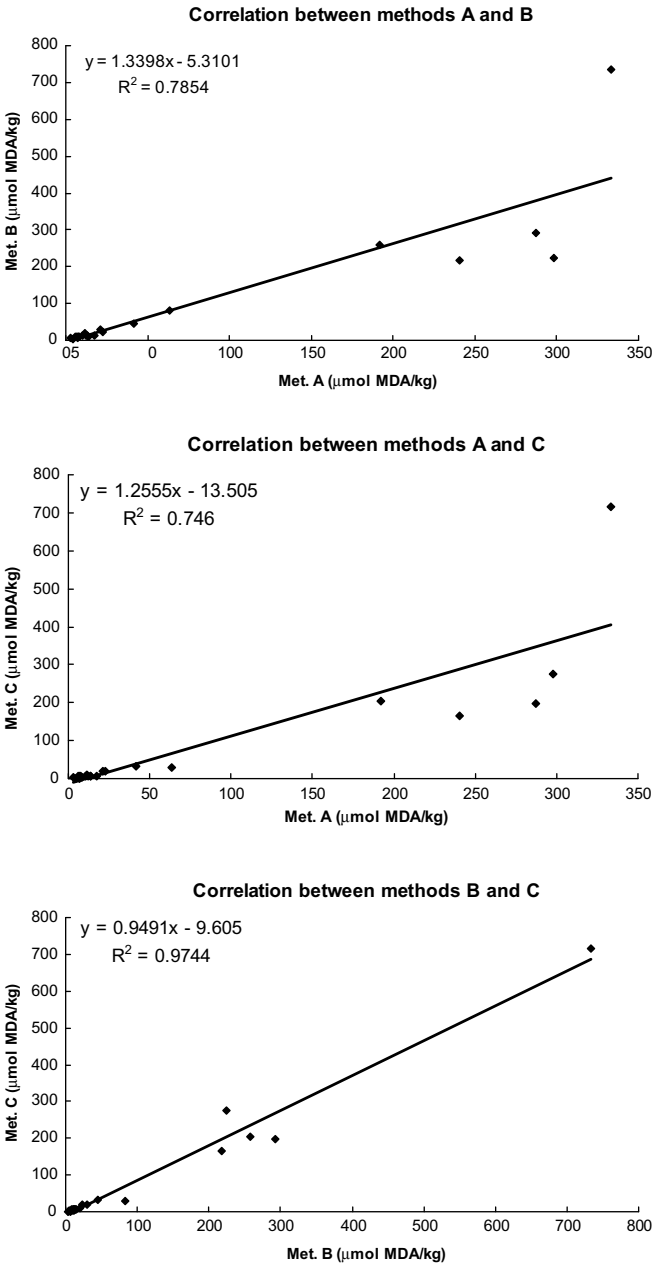

Fig. 3. Correlations between MDA levels (μmol MDA/kg) determined by methods A, B and C in hake, sea bream and sardine samples during chilled storage at 2 ± 1 ± °C.

that method A had a great deviation with the sardine sample of day 15, which also explains correlation slopes above the unity (Fig. 3).

4. Conclusions

Methods for MDA determination using HPLC separation after TBA or DNPH derivatisation were developed for routine fish analysis and optimized. Overall method performance based on accuracy and specificity followed the order, HPLC method MDA-DNPH > HPLC method MDA-TBA > Traditional spectrophotometric TBA test. For sample extraction it was found that TCA deproteinization must be done before the addition of derivatising reagent in order to ensure a better MDA extraction. The present study showed that in fish samples both developed HPLC methods are fast, simple, sensitive and stable, as shown by short retention and elution times (not exceeding 20 min), linearity, acceptable limits of detection and quantification, inter- and intra-assay CV precision testing and recovery assay. On the other hand, the traditional spectrophotometric TBA test presented low recovery levels (under 71%). Regarding accuracy and specificity for MDA, methods A and B (MDA-TBA) were inferior to method C (MDA-DNPH) as a result of possible artefactual peroxidation of sample constituents, namely, polyunsaturated fatty acids and, also, of TBA reactivity with other components besides MDA. However, method C had some disadvantages, a relatively high limit of detection (0.20 μM MDA) and a lower reproducibility at lower MDA contents in standards and samples. Nevertheless, these are not critical drawbacks for an application in routine fish analysis, given the high MDA concentrations in oxidised fish. For a finer understanding of lipid oxidation in

fish, MDA determination by method C should be preferred to TBA techniques that only give a rough estimate of lipid oxidation in fish, encompassing thiobarbituric acid reactive substances and artefactual MDA. The application of the modified HPLC methods in fish samples with different levels of MDA showed that these methods are useful for samples with low amounts of oxidation products, as well, for samples with high levels of oxidation.

Acknowledgment

This study was financial supported by EU-QCA III-MARE/FEDER: Project "Quality and Innovation of Fishery Products".

References

Aubourg, S. P. (1999). Lipid damage detection during the frozen storage of an underutilized fish species–denaturation of fish proteins during frozen storage: Role of formaldehyde. *Food Research International, 32*(6), 497–502.

Aubourg, S. P., Sotelo, C. G., & Gallardo, J. M. (1997). Quality assessment of sardines during storage by measurement of fluorescent compounds. *Journal of Food Science., 62*(2), 295–304.

Bergamo, P., Fedele, E., Balestrieri, M., Abrescia, P., & Ferrara, L. (1998). Measurement of malondialdehyde levels in food by high-performance liquid chromatography with fluorometric detection. *Journal of Agricultural and Food Chemistry, 46*, 2171–2176.

Bird, R. P., Hung, S. S. O., Hadley, M., & Draper, H. H. (1983). Determination of malonaldehyde in biological materials by high-pressure liquid chromatography. *Analytical Biochemistry, 128*, 240–244.

Careche, M., & Tejada, M. (1988). Interference by formaldehyde in the 2-thiobarbituric acid test for rancidity. *Journal of the Science of Food and Agriculture, 43*, 49–58.

Cesa, S. (2004). Malonaldehyde contents in infant milk formulas. *Journal of Agricultural and Food Chemistry, 52*, 2119–2122.

Chaijan, M., Benjakul, S., Visessanguan, W., & Faustman, C. (2006). Changes of lipids in sardine (*Sardinella gibbosa*) muscle during iced storage. *Food Chemistry, 99*, 83–91.

Cighetti, G., Debiasi, S., Paroni, R., & Allevi, P. (1999). Free and total malondialdehyde assessment in biological matrices by gas chromatography–mass spectrometry: what is needed for an accurate detection. *Analytical Biochemistry, 266*(8), 222–229.

De las Heras, A., Schoch, A., Gibis, M., & Fischer, A. (2003). Comparison of methods for determining malondialdehyde in dry sausage by HPLC and the classic TBA test. *European Food Research and Technology, 217*, 180–184.

Draper, H. H., & Hadley, M. (1990). Malondialdehyde determination as index of lipid peroxidation. *Methods in Enzymology, 186*, 421–431.

Ekström, T., Garberg, P., Egestad, B., & Högerg, J. (1988). Recovery of malondialdehyde in urine as a 2,4-dinitrophenylhidrazine derivative analysed with high-performance liquid chromatography. *Chemico-Biological Interactions, 66*(3-4), 177–187.

Fenaille, F., Mottier, P., Turesky, R. J., Ali, S., & Guy, P. A. (2001). Comparison of analytical techniques to quantify malondialdehyde in milk powders. *Journal of Chromatography A, 921*, 237–245.

Fernández, J., Pérez-Álvarez, J. A., & Fernández-López, J. A. (1997). Thiobarbituric acid test for monitoring lipid oxidation in meat. *Food Chemistry, 59*(3), 345–353.

Guillen-Sans, R., & Guzman-Chozas, M. (1998). The thiobarbituric acid (TBA) reaction in foods: a review. *Critical Reviews in Food Science and Nutrition, 38*(4), 315–330.

Jardine, D., Antolovich, M., Prenzler, P. D., & Robards, K. (2002). Liquid chromatography–mass spectrometry (LC–MS) investigation of the thiobarbituric acid reactive substances (TBARS) reaction. *Journal of Agricultural and Food Chemistry, 50*, 1720–1724.

Kakuda, Y., Stanley, D. W., & Van de Voort, F. R. (1981). Determination of TBA number by high-performance liquid chromatography. *Journal of the American Oil Chemistry Society, 58*, 773–776.

Ke, P. J., Cervantes, E., & Robles-Martinez, C. (1984). Determination of thiobarbituric acid reactive substances (TBARS) in fish tissue by an improved distillation spectrophotometric method. *Journal of the Science of Food and Agriculture, 35*, 1248–1254.

Ke, P. J., & Woyewoda, A. D. (1979). Microdetermination of thiobarbituric acid values in marine lipids by a direct spectrophotornetric method with a monophasic reaction system. *Analytical Chimica Acta., 106*, 279–284.

Khoschsorur, G. A., Winklhofer-Raab, B. M., Rabl, H., Auer, T., Peng, Z., & Schaur, R. J. (2000). Evaluation of a sensitive HPLC method for the determination of malondialdehyde, and application of the method to different biological materials. *Chromatographia, 52*(3-4), 181–184.

Kinter, M. (1995). Analytical technologies for lipid oxidation products analysis. *Journal of Chromatography, 671*, 223–236.

Knight, J. A., Smith, S. E., Kinder, V. E., & Pieper, R. K. (1988). Urinary lipoperoxides quantified by lipid chromatography, and determination of reference values for adults. *Clinical Chemistry, 34*(6), 1107–1110.

Mateos, R., Lecumberri, E., Ramos, S., Goya, L., & Bravo, L. (2005). Determination of malondialdehyde (MDA) by high-performance liquid chromatography in serum and liver as a biomarker for oxidative stress. Application to a rat model for hypercholesterolemia and evaluation of the effect of diets rich in phenolic antioxidants from fruits. *Journal of Chromatography B, 827*, 76–82.

Mendes, R., & Gonçalves, A. (2008). Effect of Soluble CO2 Stabilization on the Quality of Fillets from Farmed Gilthead Sea Bream (*Sparus aurata*) and European Sea Bass (*Dicentrarchus labrax*). *Journal of Aquatic Food Product Technology, 17*(4), 19–32.

Nunes, M. L., Batista, I., & Campos, R. M. (1992). Physical, chemical and sensory analysis of sardine (*Sardina pilchardus*) stored in ice. *Journal of the Science of Food and Agriculture, 59*, 37–43.

Ozden, O., Inugur, M., & Erkan, N. (2007). Effect of gamma radiation and refrigeration on the chemical and sensory properties and microbiological status of aqua cultured sea bass (*Dicentrarchus labrax*). *Radiation Physics and Chemistry, 76*, 1169–1178.

Panpipat, W., & Yongsawatdigul, J. (2008). Stability of potassium iodide and omega-3 fatty acids in fortified freshwater fish sausage. *Lebensmittel Wissenschaft und Technologie, 41*, 483–492.

Pikul, J., Leszczynski, D. E., & Kummerow, F. A. (1989). Evaluation of three modified TBA methods for measuring lipid oxidation in chicken meat. *Journal of Agricultural and Food Chemistry, 37*, 1309–1313.

Pilz, J., Meineke, I., & Gleiter, C. H. (2000). Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative. *Journal of Chromatography B, 742*(2000), 315–325.

Rodriguez-Casado, A., Carmona, P., Moreno, P., Sánchez-González, I., Macagnanoc, A., Di Natalec, C., & Careche, M. (2007). Structural changes in sardine (*Sardina pilchardus*) muscle during iced storage: Investigation by DRIFT spectroscopy. *Food Chemistry, 103*(3), 1024–1030.

Ruiz-Capillas, C., & Moral, A. (2001). Correlation between biochemical and sensory quality indices in hake stored in ice. *Food Research International, 34*, 441–447.

Ruiz-Capillas, C., Saavedra, A., & Moral, A. (2003). Hake slices stored in retail packages under modified atmospheres with CO_2- and O_2-enriched gas mixes. *European Food Research and Technology, 218*, 7–12.

Sakai, T., Habiro, A., & Kawahara, S. (1999). High-performance liquid chromatographic analysis of 1,3-diethyl-thiobarbituric acid-malonaldehyde adduct in fish meat. *Journal of Chromatography B, 726*, 313–316.

Seljeskog, E., Hervig, T., & Mansoor, M. A. (2006). A novel HPLC method for the measurement of thiobarbituric acid reactive substances (TBARS). A comparison with a commercially available kit. *Clinical Biochemistry, 39*, 947–954.

Sinnhuber, R. 0., & Yu, T. C. (1958). 2-Thiobarbituric acid method for the measurement of rancidity in fishery products II. The quantitative determination of malonaldehyde. *Food Technology, 12*, 9–12.

Sinnhuber, R. O., Yu, I. C., & Yu, T. C. (1958). Characterization of the red pigment formed in the 2-thiobarbituric acid determination of oxidative rancidity. *Food Research, 23*, 624–634.

Squires, E. J. (1990). High-performance liquid chromatographic analysis of the malondialdehyde content of chicken liver. *Poultry Science, 69*, 1371–1376.

St. Angelo, A. J. (1996). Lipid oxidation in foods. *Critical Reviews in Food Science and Nutrition, 36*, 175–224.

Stalikas, C. D., & Konidari, C. N. (2001). Analysis of malondialdehyde in biological matrices by capillary gas chromatography with electron-capture detection and mass spectrometry. *Analytical Biochemistry, 290*(8), 108–115.

Tarladgis, B. G., Pearson, A. M., & Dugan, L. Jr., (1964). Chemistry of the 2-thiobarbituric acid test for determination of oxidative rancidity in foods. II. Formation of the TBA malonaldehyde complex without acid heat treatment. *Journal of the Science of Food and Agriculture, 15*, 602–607.

Tsaknis, J., Lalas, S., & Evmorfopoulos, E. (1999). Determination of malondialdehyde in traditional fish products by HPLC. *The Analyst, 124*, 843–845.

Türközkan, N., Erdamar, H., & Seven, I. (2006). Measurement of total malondialdehyde in plasma and tissues by high-performance liquid chromatography and thiobarbituric acid assay. *Fırat Tıp Dergisi, 11*(2), 88–92.

Volpi, N., & Tarugi, P. (1998). Improvement in the high-performance liquid chromatography malondialdehyde level determination in normal human plasma. *Journal of Chromatography B, 713*, 433–437.

Vyncke, W. (1970). Direct determination of the thiobarbituric acid value in trichloroacetic extracts of fish as a measure of oxidative rancidity. *Fette Seifen anstrichm., 12*, 1084–1087.

Zipser, M. W., Kwon, T. W., & Watts, B. M. (1964). Changes in cured and uncured frozen cooked pork. *Journal of Agricultural and Food Chemistry, 12*, 101–109.